Claims

159. A method of stimulating expression of at least one defensin in a mammal in need thereof, by administering a compound comprising soluble CD14 or a polypeptide portion of CD14 that enhances said expression, or a conservatively substituted variant of said CD14 or the portion that enhances said expression.

160. The method of claim 159, wherein said administering step includes directly exposing epithelial cells of the mammal to said compound.

161. The method of claim 160, comprising exposing the tongue and/or the gastrointestinal tract, optionally including the small intestine, of a said mammal to an effective amount of a said compound.

162. The method of claim 160, comprising exposing the respiratory tract of a said mammal to an effective amount of a said compound.

163. The method of claim 160, wherein the CD14 has an amino acid sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and conservatively substituted variants of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:7.

164. A method of ameliorating the symptoms of sepsis comprising administering to a mammal in need thereof an effective amount of a soluble protein so as to directly expose epithelial cells of the mammal to the protein, the protein having an amino acid sequence which is at least about 63% conserved in relation to the amino acid sequence identified as SEQ ID NO:5 and having the ability to induce expression of defensins in epithelial cells.

165. The method of claim 164 wherein the protein has an amino acid sequence which is at least about 68% or about 71% or about 73% or about 78% or about 83% or about 88% or about 93% or about 98% conserved in relation to the amino acid sequence identified as SEQ ID NO:5.

166. A method of prophylactically treating a lipopolysaccharide-induced host inflammatory response in a mammal, which method comprises administering a therapeutically effective amount of an effective amount of a protein to the mammal so as to directly expose epithelial cells of the mammal to the protein, the protein having an amino acid sequence which is at least about 63% conserved in relation to the amino acid sequence identified as SEQ ID NO:4 or identified as SEQ ID NO:5 or identified as SEQ ID NO:6 and having the ability to enhance expression of one or more defensins in bovine epithelial cells.

167. The method of claim 166 wherein the protein has an amino acid sequence which is at least about 68% or about 71% or about 73% or about 78% or about 83% or about 88% or about 93% or about 98% conserved in relation to the amino acid sequence identified as SEQ ID NO:5.

168. A method of enhancing expression of defensins in a mammal in need thereof, by administering an effective amount of a soluble protein to the mammal, the protein having an amino acid sequence which is at least about 63% conserved in relation to the amino acid sequence identified as SEQ ID NO:4 or identified as SEQ ID NO:5 or identified as SEQ ID NO:6 and having the ability enhance expression of defensins in mammalian epithelial cells.

169. The method of claim 168 wherein the protein has an amino acid sequence which is at least about 68% or about 71% or about 73% or about 78% or about 83% or about 88% or about 93% or about 98% conserved in relation to the amino acid sequence identified as SEQ ID NO:5.

170. The method of claim 159, wherein the compound comprises CD14 obtained from a mammalian mammary secretion.

171. The method of claim 170, wherein the CD14 is obtained from bovine milk.

172. The method of claim 170 wherein, if the secretion has been previously subjected to a treatment step, the treatment step is sufficiently mild to permit preservation of CD14 activity for inducing or stimulating defensin production in epithelial cells.

173. The method of claim 170, wherein the CD14 is contained in a liquid.

174. The method of claim 173, wherein the liquid comprises a fraction of the milk enriched in said CD14.

175. The method of claim 170, wherein said CD14 is contained in an edible product.

176. The method of claim 170, including administering the CD14 to the mammal orally.

177. A method for determining the suitability of a product derived from a mammary secretion for use in inducing or stimulating defensin production in mammals, the method comprising the steps of:

providing a sample of the product; and

determining the amount of CD14 present in the sample.

178. The method of claim 177, wherein, if the secretion has been previously subjected to a treatment step, the treatment step is sufficiently mild to permit preservation of the CD14 activity for inducing or stimulating said defensin production, and/or optionally, wherein determining the amount of CD14 present in the sample includes exposing the sample to an

antibody which is specific for CD14, and ascertaining whether antibody-CD14 complex is formed in the exposing step.

179. The method of claim 176 wherein the compound is administered to an infant as a component of infant formula.

180. The method of claim 162including administering the compound in the form of an aerosol.

181. A method of preparing a medicament, a dietary source or masticable product for use in directly stimulating defensin production in a mammal, method comprising the steps of:

providing a stock solution containing protein of a mammary secretion;

separating, optionally precipitating, from the solution a concentrate comprising endogenous CD14; and

determining the concentration of CD14 in the concentrate,

wherein, the mammary secretion can comprise milk, whole milk, a protein-containing portion of whole milk, or colostrum, and/or

wherein, if the secretion has been previously subjected to a treatment step, the treatment step is sufficiently mild to permit preservation of the CD14 activity for inducing or stimulating defensin production.

182. The method of claim 181, wherein the mammary secretion is bovine.

183. The method of claim 181, wherein the solution is a liquid solution and the separating step includes salting out of proteins from the solution, and optionally, wherein determining the concentration of CD14 includes exposing a sample obtained from the concentrate to a first antibody specific for CD14 to form an antibody-CD14 complex and subsequently exposing the complex to a second antibody specific for CD14, wherein the second antibody includes a reporter molecule,

wherein determining the concentration of CD14 can include exposing a sample obtained from the concentrate to a first antibody specific for CD14 to form an antibody-CD14 complex and subsequently exposing the complex to a second antibody specific for the first antibody, wherein the second antibody includes a reporter molecule.

184. The method of claim 181, wherein the precipitating step includes increasing the salt concentration of the solution to obtain an ioinic strength at least as high as would be obtained by combining a saturated aqueous solution of ammonium sulphate with a volume of a said mammary secretion, the volume of the ammonium sulphate solution being equal to 65 percent of the total volume of the combined solutions.

185. The use of a mammary secretion in a method of preparing a medicament, a dietary source or masticable product for use in directly stimulating defensin production in a mammal, method comprising the steps of:

providing a composition containing protein of the mammary secretion;

exposing the composition to an antibody which is specific for CD14; and

determining whether CD14 endogenous to the secretion is present in the sample based on whether CD14-antibody complex has formed in the exposing step.

186. The method of claim 185 wherein, if the secretion has been previously subjected to a treatment step, the treatment step is sufficiently mild to permit preservation of the CD14 activity for inducing or stimulating defensin production, optionally comprising the further step of determining the concentration of CD14 in the sample.

187. The method of claim 159, wherein the polypeptide is contained in concentrated milk.

188. The method of claim 159, including the step of direct topical exposure of the epithelium of the trachea to the polypeptide or protein, as the case may be.

189. The method of claim 159, including the step of topical exposure to the outer epidermis of a mammal, particularly to wounds thereof.

190. A method of preparing an ointment for direct topical application to a wound of human skin for ameliorating the effects of infection, particularly bacterial infection, thereof, comprising incorporating into the ointment an effective amount of a concentrate comprising CD14 obtained from a mammary secretion, or a polypeptide of claim 159.

191. A dietary source such as infant formula, milk or other liquid having added thereto a fraction of a milk product, the fraction including a higher concentration of CD14 than occurs naturally in the unfractionated milk product, wherein the milk product is one which has not been treated by a process which denatures the CD14 contained therein to the extent that CD14 loses the desired activity, particularly the ability to stimulate defensins in epithelial cells.

192. The method of claim 166, wherein the mammal is in need of protection against a microbial pathogen selected from the group consisting of virus, bacteria, fungus and yeast.

193. The method of claim 159, where the mammal a human suffering from immune deficiency.

194. The method of any of claims 159, wherein the defensin(s) is selected from the group consisting of RtNP1, RtNP2, RtNP3, RtNP4, HNP1, HNP2, and HNP3 and any combination thereof, or the group consisting of HNP1, HNP2, and HNP3.

195. A method of directly activating B cells using a soluble polypeptide having the amino acid sequence selected from the group consisting of leu-leu-leu-leu-leu-leu-pro-ser; leu-leu-leu-leu-leu-leu-pro-leu; and leu-leu-leu-leu-leu-leu-val-his, and which is specifically recognized by the monoclonal antibody 3C10 and which activates B cells, by administering to a mammal in need thereof an effective amount of said polypeptide.

Version with Markings to Show Changes Made.

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The current paradigm posits the involvement of the constitutively expressed plasma protein, lipopolysaccharide-binding protein (LBP), which forms high affinity complexes with LPS (Schumann, R.R. *et. al.* 1990. *Science* 249:1429; Wright, S.D. *et. al.* 1990. *Science* 249:1431; Wright, S.D. *et. al.* 1989. *J. Exp. Med.*170:1231). LBP is a plasma glycoprotein produced by the liver, present constitutively in plasma of healthy adult humans at 5-~~10Tg/ml~~ **10μg/ml**, which has been shown to increase in concentration up to 20-fold after an acute phase response (Schumann, R.R. *et. al.* 1990. *Science* 249:1429; Tobias, P.S. *et. al.* 1992. *Cell. Mol. Biol.* 7:239; Tobias, P.S., Mathison, J.C. and R.J. Ulevitch 1988. *J. Biol. Chem.* 263:13479; Tobias, P.S., Soldau, K. and R.J. Ulevitch 1986. *J. Exp. Med.* 164:777; Wright, S.D. *et. al.* 1990. *Science* 249:1431; Wright, S.D. *et. al.* 1989. *J. Exp. Med.*170:1231). Upon binding LBP, the ability of LPS to stimulate cytokine production in macrophages and monocytes is enhanced (Mathison, J.C., Tobias, P.S. and R.J. Ulevitch 1991. *Pathobiology* 59:185; Schumann, R.R. *et. al.* 1990. *Science* 249:1429; Wright, S.D. *et. al.* 1990. *Science* 249:1431; Wright, S.D. *et. al.* 1989. *J. Exp. Med.*170:1231).

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"Stringent hybridization conditions" takes on its common meaning to a person skilled in the art here. Appropriate stringency conditions which promote nucleic acid hybridization, for example, 6x sodium chloride/sodium citrate (SSC) at about 45°C are known to those skilled in the art. The following examples are found in Current Protocols in Molecular Biology, John Wiley & Sons, NY (1989), 6.3.1-6.3.6: For 50 ml of a first suitable hybridization solution, mix together 24 ml formamide, 12 ml 20x SSC, 0.5 ml 2 M Tris-HCl pH 7.6, 0.5 ml 100x Denhardt's solution, 2.5 ml deionized H_2O, 10 ml 50% dextran sulfate, and 0.5 ml 10% SDS. A second suitable hybridization solution can be 1% crystalline BSA (fraction V), 1 mM EDTA, 0.5 M Na_2HPO_4 pH 7.2, 7% SDS. The salt concentration in the wash step can be selected from a low stringency of about 2x SSC at 50°C to a high stringency of about 0.2x SSC at 50°C. Both of these wash solutions may contain 0.1% SDS. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions, at about ~~65□C~~ **65°C**. The cited reference gives more detail, but appropriate wash stringency depends on degree of

homology and length of probe. If homology is 100%, a high temperature (65°C to 75°C) may be used. If homology is low, lower wash temperatures must be used. However, if the probe is very short (<100bp), lower temperatures must be used even with 100% homology. In general, one starts washing at low temperatures (37°C to 40°C), and raises the temperature by 3-5°C intervals until background is low enough not to be a major factor in autoradiography.

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Figure 1A shows the differential inhibition of native human LAIT (Hu-LAIT, nhCD14) mediated B cell activation by the CD14 specific mAbs 3C10 and MEM-18 (Todd S.-C. Juan, *et. al.* 1995. *J. Biol. Chem.* 270:5219). The indicated concentration of mAbs 3C10 (——), MEM-18 (— — —) or their isotype non-specific mAbs, 12CA5 ($IgG2_b$) (----) (J. Field, *et. al.* 1988. *Mol. Cell. Biol.* 8:2159), and W3/25 (IgG_1) (— - — - —) (A.F. Williams, 1977. *Cell* 12:663), respectively, was added to 0.2 ml of serum free culture medium in a 96 well flat bottomed culture plate (Costar), which contained either: ~~1 Tg/ml~~ **1 µg/ml** of native (n) Hu-LAIT (■)or 5 ~~Tg/ml~~ **µg/ml** LPS (●). Following 5 hours of incubation at 37°C, 1.5 x 10^5 high buoyant density mouse splenic B cells, isolated as previously described (Ratcliffe, M.J. *et. al.* 1983. *J. Immunol.* 131:581), were added to each of the culture wells. At 40 hours, cultures were pulsed with 1 ~~TCi~~ µCi of ^{3}H-TdR, harvested onto filter mats 6 hours later, and thymidine uptake assessed by scintillation spectroscopy. Results are expressed as a percentage of the response induced by each of the two stimuli in the absence of any mAbs. The background response in the absence of stimulus ranged from 0.7 to 1.7 x 10^3 cpm; the responses to 5 ~~Tg/ml~~ **µg/ml** LPS and 1 ~~Tg/ml~~ **µg/ml** nHu-LAITin the absence of mAbs were 77.8 x 10^3 cpm, and 82.3 x 10^3 cpm, respectively. The error bars indicate one standard deviation about the mean of triplicate cultures.

Figure 1B shows the inhibition of recombinant (r) Hu- and rBo-LAIT mediated B cell activation by the CD14 specific mAb, 3C10. The indicated concentration of mAb 3C10, or an $IgG2_b$ isotype non-specific, mAb OX40 (Paterson, D.J. *et. al.* 1987. *Mol. Immunol.* 24:1281), was added to 0.2 ml of serum free culture medium in a 96 well flat bottomed culture plate (Costar), which contained either: 15 ~~Tg/ml~~ **µg/ml** of soluble recombinant human CD14 (rHu-LAIT) (●); 2 ~~Tg/ml~~ **µg/ml** soluble recombinant bovine CD14 (rBo-LAIT) (~~▲~~) (□), or 5 ~~Tg/ml~~ **µg/ml** LPS (○) (~~•~~). Following a 5 hour incubation at

37°C, 1.5 x 10^5 high buoyant density mouse splenic B cells, isolated as previously described (Ratcliffe, M.J. *et. al.* 1983. *J. Immunol.* 131:581), were added to each of the culture wells. At 40 hours, cultures were pulsed with 1 ~~TCi~~ **µCi** of ^{3}H-TdR, harvested onto filter mats 6 hours later, and thymidine uptake assessed by scintillation spectroscopy. Results are expressed as a percent of the response induced by each of the three stimuli in the absence of any mAb. The background response in the absence of stimulus ranged from 0.7 to 1.7 x 10^3 cpm; the responses to 5 ~~Tg/ml~~ **µg/ml** LPS,15 ~~Tg/ml~~ **µg/ml** rHu-LAIT, and 2 ~~Tg/ml~~ **µg/ml** rBo-LAIT, in the absence of mAb 3C10, were 77.8 x 10^3 cpm, 10.9 x 10^3 cpm, and 82.3 x 10^3 cpm, respectively, with a standard deviation among replicate cultures of <10%. The inhibition mediated by OX40 was less than 15% at all of the concentrations tested.

Figures 2A and 2B illustrate a comparative analysis of LPS and rBo-LAIT induction of membrane Igκ expression (mIgκ) in the murine pre-B cell line, 70Z/3. 8 x 10^4 70Z/3 cells were cultured in 0.1 ml of serum free medium in flat bottomed 96 well culture plates (Costar) for 20 hours in the presence of no stimulus, or the indicated concentrations of rBo-LAIT (~~△~~) (□), LPS derived from S. typhimurium (Sigma) (○) (~~•~~), or deep rough LPS derived from E. coli mutant D31m4 (●) (Kirkland, T.N.,Qureshi, N. and K. Takayama 1991. *Inf. and Imm.* 59:131). Cells were harvested and stained with fluorescein conjugated mAb 187.1(^{F}187.1) (Yelton, D.E. *et. al.* 1981. *Hybridoma* 7:5), specific for murine Igκ, and the proportion of mIgκ$^+$ cells assessed flowcytometrically using a B.-D. FACScan. The three upper histograms of Figure 2A illustrate the proportions of mIgκ$^+$ 70Z/3 cells after the 20 hour culture period at 37°C: in the absence of stimulus (left), in the presence of 3 ~~Tg/ml~~ **µg/ml** S. typhimurium LPS (middle), and in the presence of 0.1 ~~Tg/ml~~ **µg/ml** rBo-LAIT (right). Figure 2B shows the percentage of mIgκ$^+$ 70Z/3 cells induced by the indicated concentrations of the three stimuli.

Figure 3A shows the inhibition of rBo-LAIT mediated induction of mIgκ$^+$ 70Z/3 cells by mAb 3C10. The indicated concentration of mAb 3C10 was added to 0.1 ml of serum free medium containing no additional stimulus (■), 3 ~~Tg/ml~~ **µg/ml** of S. typhimurium LPS (○) (~~•~~), or 0.1 µg/ml rBo-LAIT (●), and the mixtures plated in 96 well plate (Costar) and incubated at 37°C for 2 hours. Subsequent to this pre-incubation period, 8 x 10^4 70Z/3 cells were added to each of the culture wells, followed by a 20 hour culture period at 37°C, after which the cells were harvested and stained with ^{F}187.1, and the proportion of mIgκ$^+$ cells assessed flowcytometrically using a B.-D. FACScan. Illustrated are the % Control

responses, i.e. the proportion of mIgκ$^+$ 70Z/3 cells observed in the presence of the indicated concentration of mAb 3C10 divided by the proportion of mIgκ$^+$ 70Z/3 cells observed in the absence of mAb 3C10 for each of rBo-LAIT and LPS inductions. Isotype non-specific mAb OX40 did not mediate greater than 15% inhibition when cultured at any of the concentrations at which mAb 3C10 was used for either of the two stimuli.

Figure 3B shows the differential inhibition of rHu-LAIT mediated induction of mIgκ$^+$ 70Z/3 cells by CD14 specific mAbs. The indicated concentration of mAbs 3C10 (●), MEM-18 (■), or their respective isotype non-specific mAbs, 12CA5 (○) (•) and W3/25(△) (□), were added to 0.1 ml of serum free medium containing 0.75 ~~Tg/ml~~ **µg/ml** nHu-LAIT. Following a 2 hour incubation at 37°C, 8 x 10^4 70Z/3 cells were added to each of the culture wells, followed by a 20 hour culture period at 37°C, after which the cells were harvested and stained with ^{F}187.1, and the proportion of mIgκ$^+$ cells assessed flowcytometrically using a B.-D. FACScan. Illustrated are the % Control responses, i.e. the proportion of mIgκ$^+$ 70Z/3 cells observed in the presence of the indicated concentration of mAbs divided by the proportion of mIgκ$^+$ 70Z/3 cells observed in the presence of 0.75 ~~Tg/ml~~ **µg/ml** nHu-LAIT in the absence of any mAb.

Figures 4A to 4C show the effect on induction of mIgκ$^+$ in 70Z/3 cells by nBo-LAIT or by LPS of diphosphoryl lipid A derived from *Rhodopseudomonas sphaeroides* (RSDPLA). 8 x 10^4 70Z/3 cells were cultured in 0.1 ml of serum free medium containing 10 ~~Tg/ml~~ **µg/ml** RSDPLA in 96 well plates (Costar), at 37°C for 2 hours. Subsequent to this pre-incubation period, the indicated concentration of ReLPS (△) (□), Figure 4A), or native Bo-LAIT (○ (•, Figure 4B) was added, followed by a 20 hour culture period at 37°C. The cells were harvested and stained with ^{F}187.1, and the proportion of mIgκ$^+$ cells was assessed flowcytometrically using a B.-D. FACScan. Two controls were run for comparison to results obtain with each stimulus. RSDLPA (▲) was added at the concentration indicated to cells which had been similarly pre-treated with RSDPLA. ReLPS was added to cells that had been similarly pre-incubated but with no RSDLPA (■, Figure 4A). n-Bo-LAIT was also added to cells that had been similarly pre-incubated with no RSDLPA (●, Figure 4B). In Figure 4C, replicate 96 well flat bottomed plates (Costar) were seeded with 8 x 10^4 70Z/3 cells in 0.1 ml of serum free medium containing the indicated concentration of RSDPLA for 2 hours at 37° C. Subsequent to this pre-incubation period, cultures were supplemented with no stimulus (▲), 5 ~~Tg/ml~~ **µg/ml** of LPS (●), or 0.3 ~~Tg/ml~~ **µg/ml** of nBo-LAIT (■), as

indicated in Figure 4C. One plate was harvested 20 hours later, and cells were stained with phycoerythrin (PE) conjugated goat anti-mouse Igκ specific antibody (Southern Biotechnology). The proportion of mIgκ$^+$ cells was assessed using a B.-D. FACScan. Illustrated are the % control responses i.e. the proportion of mIgκ$^+$ cells observed in the presence of the indicated concentration of RSDPLA divided by the proportion of mIgκ$^+$ cells observed in the presence of the indicated stimuli in the absence of RSDPLA (left-hand vertical axis). The second plate was pulsed with 1 ~~TCi~~ **μCi** of ^{3}H-TdR 14 hours after the addition of stimuli, harvested onto filter mats 6 hours later, and thymidine uptake assessed by liquid scintillation spectroscopy (right-hand vertical axis).

Figure 5A is a diagrammatic representation of nHu-LAIT/CD14. Depicted are the two regions which characterize the epitopes recognized by mAb 3C10 (amino acids 7 to 14) and MEM18 (amino acids 57 to 65). Figure 5B is a schematic of how RSDPLA may function to inhibit Hu-LAIT mediated 70Z/3 differentiation. RSDPLA may be interacting with the LPS binding site of the Hu-LAIT protein, or RSDPLA may be interacting with the putative receptor for LAIT on the 70Z/3 cell. Some elements of LPS and LAIT-protein mediated cellular activation may be shared. Also shown is mAb MEM-18, which would block the interaction of nHu-LAIT and RSDPLA according to one of the possible modes of interaction. Figure 5C shows the effect of various concentrations of mAb MEM18 on induction of mIgκ$^+$ in 70Z/3 cells by nHu-LAIT in the presence of RSDPLA. 8 x 10^4 70Z/3 cells were cultured in 0.1 ml of serum free medium containing 30 ~~Tg/ml~~ **μg/ml** of RSDPLA for 2 hours at 37° C. These cultures were supplemented with 0.75 ~~Tg/ml~~ **μg/ml** of nHu-LAIT that had been pre-incubated with the indicated concentration of mAb MEM-18 for 2 hours at 37° C. After a further 20 hour incubation at 37°C, the cells were harvested and stained with phycoerythrin (PE) conjugated goat anti-mouse Igκ specific antibody (Southern Biotechnology). The proportion of mIgκ$^+$ cells was assessed using a B.-D. FACScan. Illustrated are the % control responses i.e. the proportion of mIgκ$^+$ cells observed in the presence of the indicated concentration of RSDPLA divided by the proportion of mIgκ$^+$ cells observed (90%) in the presence of 0.75 ~~Tg/ml~~ **μg/ml** of nHu-LAIT in the absence of RSDPLA.

Figure 6A shows the quantification of nHu-LAIT/sCD14 in paired samples of human milk and serum obtained from 9 subjects at the indicated time post partum. sCD14 was quantified using a commercially available ELISA kit (IBL, Hamburg). Results are presented as the ratio of sCD14/total protein in milk (open symbols) and serum (closed

symbols), each shape of symbol representing a different subject. Total protein was determined using a colorimetric detection system (BioRad).

Figure 6B shows an analysis of B cell growth promoting activity of heat denatured nBo-LAIT. $1.5x10^5$ high buoyant density mouse splenic B cells from conventional C57Bl/6 mice were prepared as previously described (Ratcliffe, M.J.H. and Julius, M.H. 1983. J. Immunol 131:581) and cultured in 0.2 ml of serum free medium in the presence of the indicated concentrations of stimulus. The indicated concentrations of nBo-LAIT were achieved by diluting a 10X solution which had been subjected to 99.9^0C for 10 minutes in a Perkin Elmer GeneAmp PCR system 9600, or left untreated. Subsequent to this treatment, samples were cooled on ice for 5 minutes, and added to B cell containing cultures. Cultures were pulsed with ~~1TCi~~ 1μCi of ^{3}H-TdR at 40 hours, harvested 6 hours later, and thymidine uptake assessed by liquid scintillation spectroscopy.

Figures 7A -7C show the partial purification of bioactive nBo-LAIT/CD14 using a combination of sequential salting out and size exclusion chromatography. Bovine milk whey was prepared and salted out as described in the text. Shown in Figure 7A is the CD14 specific immunoblot of clarified milk whey (CM), affinity purified nBo-LAIT (nBo), and each of the $(NH_4)_2SO_4$ fractions tested. The immunoblot was carried out as described below for Figure 7D. Shown in Figure 7B is the resolution of the proteins in each of the fractions described in Figure 7A using 10% SDS-PAGE followed by silver staining. The 62% $(NH_4)_2SO_4$ fraction containing the highest proportion of nBo-LAIT/CD14 was subjected to molecular sieving on a Superdex-75 size-exclusion FPLC column (Pharmacia) equilibrated in TN buffer (10mM Tris pH 8.0, 150 mM NaCl). TN buffer was used to elute proteins at a flow rate of 0.4 ml/min, and 0.2 ml fractions were collected over a period of 40 minutes using an OD_{280} nm detector to monitor the elution profile of protein. Each of the fractions obtained was assessed for nBo-LAIT/sCD14 by immunoblot as described for figure 7D. Fractions 47 to 49 from this separation procedure contained the highest concentration of nBo-LAIT/sCD14 by immunoblot analysis. Figure 7C shows a comparative analysis of the induction of mIgκ expression in the murine pre-B cell line, 70Z/3, by affinity purified nBo-LAIT/sCD14 (nBo-LAIT). The 62% $(NH_4)_2SO_4$ fraction (○) ~~(•)~~ used as the starting material for molecular sieving; and fractions 47 (■), 48 (▲) (—), and 49 (●) isolated from the Superdex-75 that contained the peak content of nBo-LAIT/sCD14 as assessed by immunoblot analysis. 8×10^4 70Z/3 cells were cultured in 0.1 ml of serum free medium in flat bottomed

96 well culture plates (Costar) for 20 hours in the presence the indicated concentrations of each of the stimuli. Cells were harvested and stained with PE conjugated goat anti-mouse Igκ specific antibody (Southern Biotechnology), and the proportion of mIgκ$^+$ cells assessed flowcytometrically using a B.-D. FACScan.

Figure 7D shows the comparative B cell stimulatory activity of nBo-LAIT affinity purified from bovine colostrum and milk. Clarified colostral and milk whey were subjected to sequential salting out using increasing concentrations of NH_4SO_2 as described for Figure 7A. The 62% $(NH_4)_2SO_4$ fraction was solubilized and desalted and sCD14 was affinity purified on mAb 3C10 conjugated to Sephadex 4B. The affinity purified material from colostrum (○) ~~(▲)~~, and milk (●), was added at the indicated concentrations to 0.2ml cultures of serum free medium containing 1.5 x. 10^5 high buoyant density splenic B cells isolated as previously described. At 40 hours, cultures were pulsed with 1 ~~TCi~~ μCi of ^{3}H-TdR, harvested onto filter mats 6 hours later, and thymidine uptake assessed by scintillation spectroscopy. The insert in Figure 7D represents an immunoblot of milk (M) and colostral (C) bovine-derived sCD14. 250 ng of protein was resolved by 10% SDS-PAGE and the protein was then transferred to a PVDF membrane. Following blocking in 5% skim milk for 1 hour, protein was revealed using a polyclonal rabbit anti-bovine CD14 in combination with horse radish peroxidase conjugated goat anti-rabbit IgG (BioRad). Signals were detected by ECL (Amersham).

Figure 8A shows the sequences of the oligonucleotide probes used for detecting mRNA specific for bovine tracheal antimicrobial peptide (TAP). Figure 8B shows the sequence of the oligonucleotide probe used to detect mRNA specific for bovine tubulin, used as a loading control.

Figure 9A shows the induction of tracheal antimicrobial peptide (TAP) mRNA in primary tracheal epithelial cells by LPS, native LAIT-protein derived from bovine (nBo-LAIT) and human (nHu-LAIT), and by recombinant bovine LAIT-protein derived from either a a mammalian expression system (rBo-C127), or a baculovirus expression system (rBo-Sf9). Primary cultures of bovine tracheal epithelial cells were prepared according to previously published methods (Diamond, G. *et.al.* 1996. *Proc. Natl. Acad. Sci. USA* 93:5156). 5 x 10^5 tracheal epithelial cells were cultured in 1 ml of serum free medium containing the indicated concentration of the various stimuli. After a 16 hour culture period at 37°C, total RNA from each of the cultures was isolated using the Trizol method (Gibco) and 20 μg loaded onto a 1.2% formaldehyde/agarose gel. Resolved RNA was then transferred to a nylon membrane

(GeneScreen, DuPont) using a Vacuum blotter (Pharmacia) in 10xSSC and UV-crosslinked according to the manufacturer recommendations. 5'-end labeled TAP oligo-probes (see Figures 8A and 8B) were mixed 1:1 and hybridized to immobilized RNA in 50% (vol/vol) formamide/6X standard saline citrate (SSC)/5X ~~Denhardt□s~~ **Denhardt's**/0.5% (wt/vol) SDS/10% (wt/vol) Dextran sulfate/100 ~~Tg/ml~~ **µg/ml** Salmon sperm DNA at 42°C for 16 to 20 hours and then washed in 2X SSC, 0.1% SDS at 65°C for 30 minutes. Loading was normalized by assessing levels of bovine tubulin in each lane. Hybridization with bovine tubulin specific oligo-probe was done using high stringency washing conditions consisting of 0.1X SSC, 1% SDS at 65°C for 2 hours. Signal intensities for TAP were normalized to relative RNA amount measured by assessing the signal intensity of the loading control probe using a PhosphorImager (Molecular Dynamics).

Figure 9B shows the kinetics of LPS and nBo-LAIT/sCD14 induction of tracheal antimicrobial peptide (TAP) mRNA in primary tracheal epithelial cells. Primary tracheal epithelial cells were prepared and cultured as described in Figure 9A. Replicate cultures all contained either 1 ~~Tg/ml~~ **µg/ml** of LPS or 1 ~~Tg/ml~~ **µg/ml** of nBo-LAIT/sCD14. At the indicated time points total RNA was isolated, resolved on agarose gels, and probed first with TAP specific oligo-probes, followed by a tubulin specific oligo probe, as described in Figure 9A. Signal intensities for TAP were normalized to relative RNA amount measured by assessing the signal intensity of the loading control probe using a PhosphorImager (Molecular Dynamics).

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The question follows as to whether mCD14 independent pathways involved in LPS and LAIT/sCD14 activation of 70Z/3 share signalling elements. The results shown in Figure 4 indicate that this might be the case. It has previously been demonstrated that diphosphoryl lipid A derived from LPS inhibits the activation of 70Z/3 by LPS (Kirkland, T.N., Quershi, N. and Takayama, K. 1991. *Infection and Immunity* 59:131). In particular, pre-incubation of 70Z/3 with diphosphoryl lipid A (RSDPLA) inhibited subsequent LPS induced expression of mIg. As shown in Figure 4A, pre-incubation of 70Z/3 in medium containing RSDPLA at 10 ~~Tg/ml~~ **µg/ml** resulted in a 3-4-fold inhibition of LPS mediated Igκ expression. Also shown in Figure 4A is that RSDPLA by itself does not induce Igκ expression on 70Z/3 over the concentration range tested, i.e., 0.1 to 30 ~~Tg/ml~~ **µg/ml**. Figure

4B illustrates that preincubation of 70Z/3 in medium containing 10 ~~Tg/ml~~ **µg/ml** RSDPLA not only inhibits nBo-LAIT induction of Igκ expression, but does so with far greater efficacy than when using LPS as stimulus, resulting in at least a 10-fold inhibition of nBo-LAIT mediated induction over the entire concentration range of nBo-LAIT tested. Illustrated in Figure 4C is that while RSDPLA inhibits both LPS and nBo-LAIT/sCD14 induction of mIgκ expression by 70Z/3, it does not inhibit the growth of 70Z/3 at any of the concentrations tested, i.e. 0.03 ~~Tg/ml~~ **µg/ml** to 10 ~~Tg/ml~~ **µg/ml**.

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Given the possible importance of the role of soluble Hu-LAIT to newborn infants, the pattern of expression of Hu-LAIT in human females after giving birth was examined. Colostrum and milk samples were obtained from nine human subjects at various times post-partum. As it is known that serum from healthy human subjects contains between 1-5 ~~Tg/ml~~ **µg/ml** sCD14, serum samples from the aforementioned nine subjects were taken to determine whether contained sCD14 concentrations paralleled those observed in mammary secretions. CD14 was quantified using a commercially available ELISA kit, and total protein was determined using a commercially available colorimetric detection system. Results shown in Figure 6A are presented as ~~Tg~~ **µg** of CD14/mg total protein for each of the paired milk and serum samples obtained. As illustrated, human milk contains between 100-400-fold more sCD14 than does serum from the same individual. Also shown is that the enrichment of sCD14 in milk versus serum persists up to 400 days post-partum.

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The sequential salting out of proteins from clarified bovine milk whey using $(NH_4)_2SO_4$ resulted in the enrichment of native bovine LAIT-protein/sCD14 in the 62% $(NH_4)_2SO_4$ fraction (compare Figures 7A and 7B). The protein concentrations in the 62% $(NH_4)_2SO_4$ fractions derived from bovine milk whey and colostral whey are 8-15 mg/ml and 47-65 mg/ml, respectively. The concentrations of LAIT-protein/sCD14 in 62% $(NH_4)_2SO_4$ fractions derived from milk and colostrum are 1-5 ~~Tg/ml~~ **µg/ml** and 5-12 ~~Tg/ml~~ **µg/ml**, respectively. Thus, LAIT-protein/sCD14 yields from these two sources is comparable at 0.15-.0.26 ~~Tg/mg~~ **µg/mg** protein.

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Primary bovine tracheal epithelial cells were prepared according to previously described methods (Diamond, G. *et.al.* 1996. *Proc. Natl. Acad. Sci. USA* 93:5156). Wells containing approximately 5 x 10^5 epithelial cells in 1 ml of serum free medium per well of a 24 well culture plate were prepared and stimulated for 16 hours with the indicated concentrations (Figure 9A) of LPS, native forms of human and bovine LAIT-protein/sCD14, or recombinant forms Bo-LAIT/sCD14 prepared using either a mammalian expression system (C127) or a baculovirus expression system (Sf9). Bovine LAIT obtained from milk was used in the experiments. As illustrated in Figure 9A, 1 ~~Tg/ml~~ **µg/ml** of either native or recombinant LAIT/sCD14, induced comparable levels of TAP specific mRNA as did 1 ~~Tg/ml~~ **µg/ml** of LPS, and resulted in a 15-20-fold increase in the signal observed in non-stimulated cells. Also shown in Figure 9A are the signals obtained for bovine tubulin specific mRNA, which indicate that comparable amounts of RNA were loaded in each track, and which were used to normalize the TAP mRNA signals.

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Shown in Figure 9B is a comparative analysis of the kinetics with which 1 ~~Tg/ml~~ **µg/ml** of either LPS or native bovine LAIT-protein/sCD14 induce TAP mRNA in cultures of primary bovine tracheal epithelial cells. Epithelial cells were cultures as described in Figure 9A and the expression of TAP specific mRNA was assessed at the indicated time points. As illustrated, both stimuli induced peak expression of TAP mRNA, normalized to the level of tubulin mRNA (Figure 9B), at 16 hours.

As far as treatments are concerned, it is important that the desired CD14 activity be retained in the concentrated CD14. Thus, in producing CD14 for use in activating B cells, a solution containing CD14 would not be heated above ~~99□C□~~ **99°C** for 10 minutes, for example, as these conditions are known to severely reduce this type of CD14 activity. See Figure 6B in which it is demonstrated that the activity of affinity purified n-bo-LAIT is essentially destroyed under these conditions.

Using the techniques described herein, a skilled person can readily determine conditions that are harmful to a desired activity or that are relatively benign. Thus for example, the effects of various temperatures and times can be applied (e.g., ~~40□C, 50□C, 60□C□□70□C~~ **40°C, 50°C, 60°C, 70°C**, etc.), could be applied to solutions containing CD14 for various lengths of time (for example, 1 minute, 2 minutes ....10 minutes) and the

effect on B cell stimulatory activity determined according to methods disclosed herein. Likewise, the effect of such conditions on defensin induction in epithelial cells could be readily determined using a similar scheme. The effects of other conditions, say salt effects, or other chemicals that might be used in the treatment of milk could also be readily determined. Care would then be taken to avoid exposure of CD14 to conditions found to be adverse to its desirable activities.

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Once the concentration of a given CD14 concentrate is determined, provided it is free of other undesirable components, it is then useful for incorporation into medicaments, food products, etc. in desired quantities. Thus, for example, 0.1 (5)g of a concentrate containing 100 ~~Tg~~/g µg/g of CD14 would be incorporated into a food bar which is to contain 10 (500) ~~Tg~~ µg of CD14.

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In situations involving CD14 proteins, about 250 to 300 ~~Tg~~ µg of polypeptide per kg of bodyweight of mammal can be fed, as an example, to a mammal per day based on an average daily consumption of between about 18 and 36 mls of fluid per day of fluid. It will be appreciated that consumption the by very young mammals increases over time. The dosage to be administered is based on the measured sCD14 concentration in adult breast milk of 10 to 20 ~~Tg~~ µg per ml, considering that a human infant increases its milk intake from about 0.1 l to about 1 l per day over the first six months after birth and assuming a weight ratio of about 28 between human and rat. In practice, particularly as human subjects are concerned, the daily dosage may well be from about 250 ~~Tg~~ µg to about 2500 ~~Tg~~ µg or more per kg of bodyweight per day. More preferably, the dosage would be in the neighborhood of from about 300 ~~Tg~~ µg to about 1 mg per kg of bodyweight per day. It may be that the preferred frequency of administration would be greater or less than once per day, depending upon the route of administration, convenience, and the variation of effectiveness of treatment with frequency of and amount used per administration. The dosage administered also depends on the subject and to which effect such administration is to give. The dosage of any one or more of the compounds will depend on many factors including the specific compound or combination of compounds being utilized, the mode of administration, and the mammal being treated. Dosages of a particular compound or combination of compounds can be determined

using conventional considerations; for example, by customary comparison of the differential activities of the subject compounds and that of a known agent, that is, by means of an appropriate pharmacological protocol.